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(71) Applicant *(for all designated States except US)*: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : MILICH, David, R. [US/US]; 11649 Vernette Court, El Cajon, CA 92020 (US). MARUYAMA, Toshiyuki [JP/US]; Bunkyo-ku, Koraku 2-21-12-5-3, Tokyo 112 (JP). THORNTON, George, B. [US/US]; 16730 Rockin Oaks Way, Ramona, CA 92065 (US).

(74) Agents: FITTING, Thomas et al.; The Scripps Research Institute, 10666 North Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).

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(54) Title: METHODS FOR THE DETECTION OF ANTIGEN-SPECIFIC IMMUNE COMPLEXES

(57) Abstract

A simple and sensitive method is described for the detection of circulating immune complexes (IC's) in an antigen-specific manner. The method is based on the use of anti-peptide antibodies as solid-phase capture reagents to bind antigen which is complexed to serum antibodies. The bound serum antibody is detected with a labelled second antibody. The method requires that the anti-peptide antibodies bind native protein efficiently, and that the anti-peptide antibodies do not compete with antibodies raised against the native protein which are involved in IC formation. Two anti-peptide antibodies specific for the hepatitis B surface antigen (HBsAg) and the hepatitis B e antigen (HBeAg), which possessed the requisite characteristics, were chosen as models for IC assay development. The solid-phase, anti-peptide based assays efficiently detected HBsAg and HBeAg-containing IC's in preformed antigen/antibody mixtures and in the serum of chronically infected hepatitis B patients.

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METHODS FOR THE DETECTION OF ANTIGEN-SPECIFIC IMMUNE COMPLEXES

Technical Field

The present invention relates to immunological methods for the detection of circulating immune complexes through the use of antibodies as solid-phase capture reagents that bind antigen complexed to serum antibodies.

Background

Historically, assays developed to measure circulating immune complexes (IC's) have emphasized detection of the immunoglobulin (Ig) component and consequently are antigen nonspecific (i.e., Clq binding, Raji cell assay, C3 binding). Antigen-specific IC assays are generally too complex for routine clinical performance.

A unique characteristic of HVB infection is the secretion into the serum of excess subviral proteins, which attain serum concentrations far greater than the virion itself. See, Heermann et al., "Surface Proteins of Hepatitis B Viruses". In: A. McLachlan (Ed.), Molecular Biology of the Hepatitis B Virus, CRC Press, pp 109 (1991). These secreted viral proteins may function in evading the anti-viral immune response during chronic infection. See, Milich et al., Proc. Natl., Acad., Sci., USA, 87:6599 (1990) and Milich et al., Proc. Natl., Acad., Sci., USA, 88:4348 (1991).

Additionally, the presence of subviral proteins including the hepatitis B surface antigen (HBsAg) and the hepatitis B e antigen (HBeAg) in the serum affect the ability to detect circulating antibodies and may obscure the onset of seroconversion. The available commercial assays usually detect anti-HBs and anti-HBe antibodies only after the respective antigens have been cleared from the serum. In HBeAg-expressing transgenic mice, the observation was recently made that anti-HBe antibodies could be detected even in the presence of HBeAg using a direct binding antibody assay [Milich et al., Proc. Natl., Acad., Sci., USA, 87:6599 (1990)]. However, detection of anti-HBe in the presence of a vast excess of serum HBeAg or detection of HBeAg in the presence of an antibody excess was more problematic.

A number of investigators have reported the presence of circulating immune complexes in patients with HBV infection. Both antigen non-specific (U.S. Patent Nos. 4,757,024 and 4,753,893, Abrass et al., Clin. Exp. Immunol., 40:292 (1980); Anh-Tran et al., J. Immunol. Methods, 33:293 (1980); Anh-Tran et al., Clin. Exp. Immunol., 43:246 (1981); and Thomas et al., Clin. Exp. Immunol., 31:150 (1978) and antigen-specific (Sallberg et al.,Clin. Exp. Immunol., 84:116 (1991); Rath et al., Clin. Exp. Immunol., 72:164 (1988); Sagnelli et al., Liver, 5:205 (1985); Brown et al., Immunol., 49:673 (1983); Brown et al., Clin. Exp. Immunol., 55:355 (1984); and Pernice et al., J. Immunol. Methods, 28:33 (1979) methods of IC detection have been utilized. The obvious limitation of the non-specific IC assays is the lack of information regarding the viral and/or subviral origin of the antigen component of the IC. The antigen-specific IC assays are either difficult and cumbersome to perform

(ie. PEG precipitation, IC disassociation followed by soluble antigen assay) or sensitive over only a narrow range of antigen:antibody ratios (ie. solid-phase assays which utilize monoclonal or polyclonal antibodies specific for the native antigens to capture the complexed antigen). The limitation of the previously described solid-phase capture methods is that the antibodies used to capture the IC's are often crossreactive with the serum antibodies involved in IC formation and therefore compete for antigen binding. See U.S. Patent Nos. 4,617,262 and 4,464,165. These types of IC assays can detect IC's only in slight antigen excess and function best in the context of multivalent antigens (Rath et al., Clin. Exp. Immunol., 72:164 (1988).

For this reason, the method to detect circulating immune complexes in an antigen-specific manner described herein was developed to avoid these complications. The method is antigen-specific, sensitive in conditions of either antigen or antibody excess, and is simple to perform. Furthermore, the use of anti-peptide antibodies to design solid-phase IC assays is not limited to the HBV system because the method is applicable to any pathogen or auto-antigen to which non-competing anti-peptide antibody can be generated.

Anti-peptide antibodies specific for two HBV antigens, the HBeAg and HBsAg, were utilized. A consistent feature (often thought of as a failing) of antibodies raised against peptide fragments has been that a high percentage of anti-peptide antibodies which recognize the native protein often bind at unique sites not relevant to the sites recognized by antibodies raised to the intact protein. See, Milich, "Synthetic T and B Cell Recognition Sites:

Implication for Vaccine Development". Advances In Immunology. Academic Press, pp 195 (1989). This characteristic of anti-peptide antibodies is advantageous since it makes them ideal candidates to function as capture reagents for antigens already complexed to serum antibodies directed to the native protein. Therefore, anti-peptide antibodies that bind native HBeAg or HBsAg were selected, but that do not compete with antibodies raised against the native antigens, to capture these serum antigens on a solid-phase whether the antigen is free or immune-complexed. The presence of Ig bound to the captured antigen is determined by probing with a peroxidase-labelled monoclonal anti-human IgG reagent.

Brief Summary of the Invention

The present invention provides a method of assaying circulating IC's based on the use of solid-phase anti-peptide antibodies by a simple antigen-specific method.

Therefore, the present invention describes a method for detecting the presence of an immunocomplex of antibody molecules immunocomplexed with a preselected antigen in a sample. The immunocomplex typically represents circulating immunocomplexes in a patient experiencing an immune response, the detection of which immunocomplex provides a clinician with important diagnostic information regarding the health of the patient.

The method comprises the steps of:

a) admixing the sample with a detecting antibody molecule to form an immunoreaction admixture, where the detecting antibody molecule is capable of immunoreaction with the immunocomplex at a preselected site of the immunocomplex;

b) maintaining the immunoreaction admixture under immunoreaction conditions sufficient for the detecting antibody molecule to immunoreact with the immunocomplex and form a detecting antibody-immunocomplex reaction product; and

c) detecting the presence of the detecting antibody-immunocomplex reaction product, and thereby the immunocomplex.

In preferred embodiments, the method is practiced in a solid phase format, with the detecting antibody attached to a solid support, thereby allowing the rapid detection of immunocomplexes.

In preferred embodiments, the sample is a human body fluid sample, typically blood or serum.

In preferred methods, the invention detects immunocomplexes having a preselected antigen selected from the group of antigens consisting of bacterial, viral, parasitic, fungal, tumor and self antigens. Preferred are the viral antigens selected from the group of viruses consisting of hepatitis B virus (HBV), human immunodeficiency virus, influenza A virus, Epstein-Barr virus, togavirus and rubella virus. Particularly preferred are the HBV antigens HBsAg and HBeAg.

In one embodiment, the method uses a detecting antibody which is directed to a preselected epitope on the antigen defined by a polypeptide. In this embodiment, the detecting antibody is an anti-polypeptide antibody. Preferred are anti-polypeptide antibodies that immunoreact with the hepatitis B virus (HBV) antigen HBsAg and immunoreact with a polypeptide having an amino acid residue sequence shown in SEQ ID NO 1, or that immunoreact with the hepatitis B virus (HBV) antigen HBeAg and immunoreact with a polypeptide having an amino acid residue sequence shown in SEQ ID

NO 2.

Brief Summary of the Drawings

In the drawings forming a portion of this disclosure:

Figure 1 illustrates the solid-phase anti-peptide specifically binds soluble native antigens. The IgG fractions of either anti-HBs 125-137 (Figure 1A) or anti-HBe 73-87 (Figure 1B) antisera were coated on the solid-phase and dilutions of soluble HBsAg (open circles) or HBeAg (closed circles) were added. Bound soluble antigen was detected by peroxidase-labelled human anti-HBs or human anti-HBe, and is expressed as absorbance (OD_{492}) corrected for absorbance in the absence of antigen.

Figure 2 illustrates the kinetics of antibody seroconversion and IC formation in a case (N.Y.) of resolving chronic hepatitis B infection (Figure 2A, upper panel). Serum samples were collected from 3/81 through 6/83 and analyzed for HBV DNA (dot hybridization); HBeAg, HBsAg, a-HBe and a-HBs (commercial assays, Abbott); and ALT levels (unit/l). The same samples were assayed for anti-HBs (open circles) by direct EIA and HBsAg/ICs (closed circles) in the antigen-specific IC assay using anti-HBs 125-137 as the capture antibody shown in Figure 2B, middle panel; and anti-HBe (open squares) by direct EIA and HBeAg/ICs (closed squares) in the antigen-specific IC assay using anti-HBe 73-87 as the capture antibody. The data are expressed as a P/N ratio, which represents the absorbance (OD_{492}) value of the sample (P) as compared to the mean absorbance value of 20 negative control sera (N).

Figure 3 illustrates the kinetics of antibody seroconversion and IC formation in a case (E.I.) of

resolving chronic hepatitis B infection (Figure 3A, upper panel). Serum samples were collected from 1975 through 1983 and analyzed for HBV DNA (dot hybridization); HBeAg, HBsAg, a-HBe and a-HBs (commercial assays, Abbott); and ALT levels (unit/l). The same samples were assayed for anti-HBe (open squares) by direct EIA and HBeAg/ICs (closed squares) in the antigen specific IC assay using anti-HBe 73-87 as the capture antibody. The data are expressed as P/N ratio, which represents the absorbance (OD_{492}) value of the sample (P) as compared to the mean absorbance value of 20 negative control sera (N).

Detailed Description of the Invention

A. Definitions

"Amino acid residue" refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 CFR 1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	

Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
J	Xaa	Unknown or other

The term "antibody" in its various grammatical forms is used herein as a collective noun that refers to a population of immunoglobulin molecules and/or immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "antibody molecule" in its various

grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F9ab')$_2$ and F(v).

Fab and F(ab')$_2$ portions of antibodies are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibodies by methods that are well known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous and Dixon. Fab' antibody portions are also well known and are produced from F(ab')$_2$ portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules are preferred, and are utilized as illustrative herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to a population of one species of antibody molecule of determined (known) antigen-specificity. A monoclonal antibody contains only one species of antibody combining site capable of immunoreacting with a particular antigen and thus typically displays a single binding affinity for that antigen. A monoclonal antibody may therefore contain a bispecific antibody molecule having two antibody combining sites, each immunospecific for a different antigen.

As used herein, the term "biological assay conditions" is sued for those conditions wherein a

molecule useful in this invention such as an antibody binds to another useful molecule such as an antigen epitope within a pH value range of about 5 to about 9, at ionic strengths such as that of distilled water to that of about one molar sodium chloride, and at temperatures of about 4 degrees C to about 45 degrees C.

The word "complex" as used herein refers to the product of a specific binding agent-ligand reaction. An exemplary complex is an immunoreaction product formed by an antibody-antigen reaction.

A "specific binding agent" is a molecular entity capable of selectively binding another molecular entity or ligand. Exemplary specific binding agents are paratopic molecules, complement proteins or fragments thereof, S. aureus protein A. and the like. Preferably, the specific binding agent binds its ligand when the ligand is present as part of a complex.

The word "antigen" is a polypeptide or protein that is able to specifically bind to (immunoreact with) an antibody and form an immunoreaction product (immunocomplex). The site on the antigen with which the antibody binds is referred to as an antigenic determinant or epitope.

B. Assay Methods

The present invention broadly contemplates a method of detecting the presence of immune complexes, preferably those antigen-specific immune complexes circulating in serum.

In accordance with this method, to detect the presence of any formed circulating immunocomplexes, a portion of the aqueous protein compositions, typically serum from a patient such as described in Example 5,

is admixed with a solid support capable of specifically binding the immunocomplex, thereby forming a solid/liquid phase (capturing reaction) admixture.

Solid supports capable of specifically binding an immunocomplex are typically comprised of a specific binding agent affixed (operatively linked) to a solid matrix. Preferably, the solid-phase affixed specific binding agent is an antibody molecule capable of immunoreacting with the immunocomplex. It should be noted that an antibody capable of immunoreacting with either the antigen or the antibody of the surface immunocomplex can be used. Preferred antibody compositions for use in the method of this invention bind to the antigen at unique sites not relevant to the sites recognized by antibodies raised to the intact protein. The preferred antibodies, either polyclonal or monoclonal antibodies, bind and capture antigens already complexed to serum or native antibodies directed to the native antigen. Preferred antibodies for use in detecting circulating the hepatitis B virus surface antigen (HbsAg) and the hepatitis B e antigen (HbeAg) are anti-peptide antibodies raised against the amino acid residue sequence from 125 to 137 of HbsAg of HbsAg of subtype adw and the amino acid residue sequence of HbeAg of subtype agw from 73 to 87, respectively. The peptides and their corresponding antibodies are described in Examples 1 and 2.

Useful solid matrices are well known in the art. Such materials are water insoluble and include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia, Piscataway, NJ; agarose; polyvinylchloride, polystyrene, cross-linked polyacrylamide, nitrocellulose or nylon-based webs

such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene, polycarbonate or polyvinylchloride.

When present as part of a solid support, a specific binding agent is typically affixed to a solid matrix by adsorption from an aqueous medium, although other modes of affixation, such as covalent coupling, well known to those skilled in the art, can be used.

The solid/liquid phase admixture is maintained for a time period sufficient for any immunocomplex present in serum or another body fluid to be bound by the solid support and thereby form a solid-phase bound complex. Preferably, the maintained solid support is then separated from any non-bound protein, typically by washing.

Assaying the maintained solid support for the presence of solid-phase bound complex thereby provides a means for detecting the presence of the immunocomplex of interest in the aqueous sample. Such assaying is typically performed by forming a labeling reaction admixture by admixing the maintained solid support with a labeled specific binding agent capable of specifically binding the immunocomplex portion of the solid-phase complex. Preferably, the labeled specific binding is capable of binding either the antigen or the antibody of the surface immunocomplex when the surface immunocomplex is itself bound to the solid support. More preferably, the solid-phase bound complex is detected with an anti-IgG antibody containing a labeling reagent, such as horseradish peroxidase.

The labeling reaction admixture is maintained for a time period sufficient for the labeled specific binding agent to react with (specifically bind) any solid-phase immunocomplex present and thereby form a

labeled solid-phase product. Any non-bound labeled specific binding agent is then typically separated from the solid support.

The presence of any labeled solid-phase product formed is then determined by known assay procedures that depend, as is well known, on the type of label used. The presence of labeled solid-phase product indicates the presence of the immunocomplex of interest in the aqueous sample.

As used herein, the term "circulating immunocomplex" means a complex formed of an antibody bound to a circulating antigen epitope whose presence is sought. The immunocomplex can be present in the aqueous sample analyzed as obtained from a patient (donor) as where the sample is a serum sample from a patient such as a patient with hepatitis B virus infection that contains autoantibodies to a hepatitis B viral secreted proteins, such as HbsAg and HbeAg. The immunocomplex to be assayed by the methods of this invention includes any immunocomplexes circulating in blood for which detection of specific antigens are desired for serological analysis, such as with other acute or chronic viral bacterial, or fungal, or parasitic infections. Thus, for detection of various immunocomplexes found in serum and the like, antibodies which recognize the immunocomplex, preferable the antigen, that do not bind at the same epitope as the serum antibodies, are required as described in the following section. Also contemplated for use in this invention is the detection of an antibody-antigen complex preformed in vitro by reacting the antibody with the antigen epitope to form soluble immunocomplexes such as though described in Examples 7 and 8.

In preferred practice, sera from patients is

collected for subsequent assaying with the methods of this invention. The serum containing immunocomplexes is thereafter affixed to a solid phase support to form a solid phase-affixed immunocomplex. The affixation can be physical adsorption, immunoreaction using solid phase-bound antibodies directed to the antibodies of the immunocomplex, such as those anti-peptide antibodies described in Example 2.

The reaction immunoreaction admixture is maintained for a time period sufficient for an immunocomplex containing the antibody and the cell surface antigen to form on the cellular surface. As is well known, the time period required for immunocomplex formation depends on a variety of factors including temperature and concentration of reactants. Typically, the time period is predetermined for a given set of reaction conditions by well known methods prior to performing the assay. Under biological assay conditions, the maintenance time period is usually from minutes to hours, such as 30 minutes to about 2 hours. The immunocomplex is thereafter assayed for as a solid phase-affixed immunocomplex.

The assay methods of the present invention utilize a solid support capable of specifically binding a solubilized immunocomplex. Useful solid supports are typically comprised of a specific binding agent affixed (operatively linked) to a solid matrix.

A specific binding agent can be linked to a label or indicating means and used to form a labeled immunoreaction product.

The terms "indicating means" and "label" are used herein to include single atoms and molecules that are capable of producing a detectable signal and of being linked to an antibody or used separately.

The indicating means can be a fluorescent labeling agent that chemically binds to antibodies or antigens without denaturing them to form a fluorochrome (dye) that is a useful immunofluorescent tracer. Suitable fluorescent labeling agents are fluorochromes such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), 5-dimethylamin-1-naphthalenesulfonyl chloride (DANSC), tetramethylrhodamine isothiocyanate (TRITC), lissamine, rhodamine 8200 sulphonyl chloride (RB 200 SC) and the like.

In preferred embodiments, the indicating group is an enzyme, such as horseradish peroxidase (HRP), glucose oxidase, or the like. Where the principal indicating group is an enzyme such as HRP or glucose oxidase, additional reagents are required to visualize the fact that a complex (immunoreaction product) has formed. Such additional reagents for HRP include hydrogen peroxide and an oxidation dye precursor such as diaminobenzidine. An additional reagent useful with glucose oxidase is 2,2'-azino-di-(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling agents and are used illustratively herein.

An exemplary radiolabeling agent is a radioactive element that produces gamma ray emissions. Elements which themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{131}I, ^{132}I, and ^{51}Cr represent one class of gamma ray emission-producing radioactive element indicating groups. Particularly preferred is ^{125}I. Another group of useful indicating groups are those elements such as ^{11}C, ^{18}F, ^{15}O and ^{13}N which themselves emit positrons. the positrons so emitted produce gamma rays upon encounters with electrons present in the animal's body. Also useful is a beta emitter, such as

^{111}indium.

The labeling of proteinaceous specific binding agents is well known in the art. For instance, antibodies produced by hybridomas can be labeled by metabolic incorporation of isotope containing amino acids provided as a component in the tissue culture medium. See for example Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978) and U.S. Patent No. 4,493,795 which is incorporated herein by reference. Method for conjugating enzymes to proteins may be found in U.S. Patent No. 3,791,932 and No. 3,839,153. In addition, site directed coupling reaction can be carried out so that the label does not substantially interfere with the immunoreaction of the second receptor with apo B-100. See, for example, Rodwell et al., Biotech., 3:889-894 (1985).

C. Immune Complexes

An immune complex (also referred to as IC) as defined for detection by use of the methods of the present invention consists of an antigen complexed with an antibody that immunoreacts with an immunodominant epitope on the antigen. Preferably the immune complex is circulating in the blood but also contemplated is an immune complex located extravascular tissues. Further contemplated for detection is an immune complex present on the surface of a cell, such as that seen in patients with subacute sclerosing panencephalitis where the patients' antibodies to the measles virus envelope glycoproteins are complexed with viral antigens on the surface of

the virally infected cell. These types of immune complexes are defined as native. Also contemplated for use with the methods described herein are immune complexes that are nonnative such as those preformed in vitro by the admixture of antigen and antibody such as those described in Examples 7 and 8.

The formation of immune complexes circulating in the blood depend on the relative proportions of antigen and antibody and also on antibody affinity. With an excess of antibody, each antigen molecule is bound with antibody and is rapidly removed by reticulendothelial cells. When equal amounts of antigen and antibody are present, lattice structures resulting in large aggregates are formed and eventually removed. However, when antigen concentration is in excess or serum-derived antibodies are of lower affinity, the formed complexes are not removed from the circulation.

Those immune complexes that remain in the circulation are formed in most acute viral infections where after virus enters the blood stream, an immune response is generated. This state persists until antibody responses increase or greater affinity antibodies combine with the antigen. Under those circumstances, the resultant complexes are deposited in blood vessels and in liver glomeruli over the course of long term viral infection causing release of viral antigens into the blood as is a unique characteristic of hepatitis B virus (HBV) infection. Subviral proteins are secreted into the serum and attain serum concentrations in excess of intact viral concentrations. Circulating immune complexes are also deposited in the walls of small blood vessels in the skin and joints where subsequent inflammatory reactions occur. Viral infections where immune

complexes are formed besides HBV and measles include Epstein-Barr virus, togavirus, human immune deficiency virus and influenza A virus.

The antibodies generated against viral, bacterial, fungal, self, parasitic or tumor-derived antigens and the like recognize the immunodominant epitope on that antigen. What is meant by immunodominant epitope is that antibodies are generally raised against and recognize the native conformation of a protein or antigen. For instance, antibodies to native staphyloccal nuclease were found to have an increased affinity of approximately 5000-fold to the native protein that for the corresponding polypeptide on which they were isolated. Thus, the specificity with the generation of the immune response of this type is dependent not only on the antigenic determinant based on a specific sequence of amino acid residue sequences but more importantly on the three dimensional conformation of those residues constrained in space. The antibodies complexed with circulating antigens such as those recognizing hepatitis B viral surface and e antigens have been shown to bind to specific sites on the antigen.

For detection of the immune complexes as performed by the methods of the present invention, the antibody reagent used for binding to the immune complex must then necessarily not be directed in specificity towards the same site as those recognized by those found in serum. More specifically, the antibody must recognize a nondominant site on the antigen comprising the immune complex. In addition, the antibody must be able to bind to the immune complex without any steric hindrance imposed by the immune complex itself. Thus, for the detection of

immune complexes, an antibody reagent having such a specificity is required. This reagent is referred to as the detecting reagent or detecting antibody. The detecting antibody thus functions as a capture reagent for the immune complexes, without the binding of which no detection would occur. Therefore detecting antibody is synonymous with capture antibody.

Contemplated for use as detecting antibodies are antibody reagents including polyclonal and monoclonal antibodies raised against intact antigen immunogens, polyclonal or monoclonal antibodies raised against preferred polypeptide sequences of the antigen; and monoclonal antibodies that recognize intact antigen or derived polypeptides prepared from recombinant combinatorial library techniques such as those prepared as described by Persson et al., Proc. Natl. Acad. Sci., USA, 88:2432-2436 (1992) and Barbas et al., Proc. Natl. Acad. Sci., USA, 88:7978-7982 (1992).

In addition to the detecting antibody having the specificity for a nondominant site on the antigen in the immune complex is a detecting antibody having the specificity for a native antibody produced in response to a foreign antigenic load. Thus, the detecting antibody is typically an anti-idiotypic antibody.

Circulating immune complexes have been identified in hepatitis B virus infections, other viral infections previously mentioned, various lung diseases (cryptogenic fibrobosing alveolitis, chronic interstitital pneumonia, fulminating pulmonary edema, and chronic bronchitis), in sarcodoisis, in several collagen-vascular diseases such as rheumatoid arthritis, in leprosy, in retinal vasculitis and in schitosomiasis to name a few. In order to detect immune complexes such as those listed herein, a detecting reagent, preferably a detecting antibody,

must be produced, and if already produced, must be screened for the ability to bind to the immune complex at a site that is not inhibited by the antibody comprising part of the immune complex. Detecting antibodies can be raised as described herein against the intact antigen in question or a specified polypeptide sequence comprising a part of the antigen. Amino acid residue sequences of typical antigens such as those listed herein are available through databases such as GenBank. Defined polypeptides can then be used as immunogens as described in Example 1 for hepatitis B virus surface and e antigens. The resultant antibodies are then screened for immunospecificity. Typical screening assays for determining the binding specificity of detecting antibodies include binding and inhibition of binding assays such as those described in Examples 5 and 6. With such an approach, any potential detecting reagent can be obtained to allow for the detection of an immune complex.

Examples

The following examples relating to this invention are illustrative and should not, of course, be construed as specifically limiting the invention. Moreover, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are to be considered to fall within the scope of the present invention hereinafter claimed.

1. Preparation of Recombinant Antigens and Synthetic Peptides

Recombinant hepatitis B surface antigen (HBsAg) of the adw subtype was provided by G. Bitter (Amgen,

Thousand Oaks, CA). Recombinant hepatitis B e antigen (HBeAg) of the ayw subtype was provided by S. Stahl (Stahl et al., Proc. Natl. Acad. Sci., USA, 79:1606 (1982); Milich et al., J. Immunol., 141:3617 (1988)). Synthetic peptides were synthesized by the Merrifield solid-phase method, and were subjected to HPLC on a C18 reverse phase column. All peptides eluted as a single major peak (>90%). Synthetic peptides were produced in the peptide laboratory of the R.W. Johnson Pharmaceutical Research Institute, La Jolla, CA, and were provided by K. Hoey. The HBsAg-derived peptide utilized is comprised of amino acids 125-137 of HBsAg/adw and is designated as HBs 125-137: TTPAQGNSMFPSC (SEQ ID NO 1) [Alexander et al., "Chemically Synthesized Peptide Analogues of the Hepatitis B Surface Antigen. In: F.V. Chisari (Ed.), Advances in Hepatitis Research, pp 223 (1984)]. The HBeAg-derived peptide utilized is comprised of amino acids 73-87 (exclusive of the precore sequence) of HBeAg/ayw and is designated as HBe 73-87: GVNLEDPASRDLVVSC (SEQ ID NO 2).

2. Preparation of Antibodies

To produce anti-peptide antibodies, peptides HBs 125-137 and HBe 73-87 were coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde. Rabbits were immunized subcutaneously with 200 μg of the peptide-KLH conjugate emulsified in complete Freund's adjuvant (CFA), and were boosted with an equal dose of peptide conjugate in incomplete adjuvant. Antisera were collected and IgG fractions were obtained by ion exchange chromatography (Bio-Rad Econo-Pac 10 DG). The concentration of anti-HBs 125-137 was 1.0 milligrams/milliliter (mg/ml) and anti-HBe 73-87 was 4.3 mg/ml.

Human polyclonal anti-HBs and anti-HBe antisera were obtained from recovered HBV infected patients. Murine polyclonal anti-HBs and anti-HBe were produced by immunization with recombinant antigens. Polyclonal rabbit anti-HBe was purchased (DAKO Corporation, CA), and the monoclonal anti-human IgG was provided by Ortho Diagnostics, NJ.

3. Solid-Phase Direct Enzyme Immunoassays

Direct solid-phase enzyme immunoassays (EIA) were used to measure free anti-HBs and free anti-HBe in serum and in antigen/antibody mixtures in vitro. Recombinant HBsAg or HBeAg diluted in 0.01 M bicarbonate buffer, pH 9.6 were coated onto microtiter plates (50 nanograms (ng) in each well) overnight at 4 degrees Celsius (4°C). The plates were washed 5x in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and .005% Tween 20 (wash buffer). The plates were preincubated for 1 hour at 37°C with 50 microliter (ul) of PBS containing 1% BSA, 0.005% Tween 20 and 5% heat inactivated goat serum (blocking buffer) to reduce nonspecific binding. The sera to be tested for anti-HBs or anti-HBe reactivity were diluted 1:50 in blocking buffer and added to the solid-phase antigen plates and incubated for 2 hours at 37°C. After washing, 50 ul of peroxidase conjugated monoclonal anti-human IgG (1:2000) was added and the plates were incubated for 2 hours at 37°C. The plates were developed by a final incubation for 10 minutes with 50 ul of orthophenylene diamine (OPD). The absorbance was read on an automatic microtiter plate reader (Dynatech; MR600) and results are expressed as optical density (O.D.) units at 492 nm. All EIA's were performed on at least three occasions, and the experiments shown are

representative.

4. Quantitative Antigen-Specific Immune Complex Assays

To detect antigen-specific immune complexes in serum or in antigen/antibody mixtures in vitro, solid-phase EIA's were developed. To detect HBsAg-containing IC's, microtiter plates were coated with an IgG fraction of anti-HBs 125-137 (0.5 microgram (ug) in each well), and to detect HBeAg-containing IC's, plates were coated with an IgG fraction of anti-HBe 73-87 (1.0 ug/well) in 0.01 M bicarbonate buffer, pH 9.6 overnight at 4°C. The plates were then washed and blocked and the sera to be tested were diluted 1/50 in blocking buffer and added to the anti-peptide coated plates. The plates were incubated for 2 hours at 37°C, washed and developed with the peroxidase-labelled Mab anti-human IgG as described.

5. Patients

Twenty patients who were consistently positive for HBsAg and HBeAg in serum for more than 1 year and were diagnosed as chronic active hepatitis (CAH) were studied. All sera had been tested for HBsAg/anti-HBs, HBeAg/anti-HBe, anti-HBc, IgM-anti-HBc, and anti-HD using commercial enzyme immunoassays (EIA's; Abbott Laboratories, IL). Hepatitis B virus DNA (HBV-DNA) was determined using a dot blot procedure. Human anti-HBs positive sera were obtained from patients who had recovered from acute hepatitis B, and human anti-HBe positive sera were obtained from patients who had been chronically infected and subsequently seroconverted to anti-HBe positive. In each assay at least 15 human sera, which were negative for all HBV

markers, were used as negative controls. The mean plus 2 standard deviations of the absorbance values of the control sera was used as the cut off value in all assays. Statistical analyses were performed using a standard chi-square (X2) test.

6. Characterization of Anti-Peptide Antibodies

In order for an anti-peptide antibody to be useful as a reagent capable of capturing circulating IC's several criteria were first satisfied. First, the anti-peptide reagent must efficiently bind the native protein antigen in a specific manner. Anti-HBs 125-137 has been shown previously to bind native HBsAg/adw [Alexander et al., "Chemically Synthesized Peptide Analogues of the Hepatitis B Surface Antigen. In: F.V. Chisari (Ed.), Advances in Hepatitis Research, pp 223 (1984)] and as illustrated in Table I efficiently binds HBsAg but not HBeAg or the particulate nucleocapsid antigen (HBcAg). The HBeAg-derived peptide 73-87 was selected from a panel of non-overlapping peptides because immunization with this sequence elicited the highest titer antibody response to native HBeAg and HBcAg (Table I). Anti-HBe 73-87 bound HBcAg as well as HBeAg because this amino acid sequence is shared between HBeAg and HBcAg, and is obviously accessible on the surface of both proteins. However, circulating immune complexes are more likely to contain HBeAg as opposed to HBcAg because HBeAg is a secreted protein whereas HBcAg accumulates intracellularly in infected hepatocytes and is only secreted as an internal component of the intact virion.

Table I. Specificity of Anti-peptide Antisera for Native Antigens

Anti-peptide	Antibody Titer		
	HBsAg	HBeAg	HBcAg
Anti-HBs 125-137	1:25,600	0	0
Anti-HBe 73-87	0	1:102,400	1:409,600

Solid-phase recombinant antigens were coated on plastic wells (50 ng/well) and were incubated with 4-fold dilutions of the rabbit anti-peptide antisera. Titers are expressed as the highest dilution to yield 3x the OD_{492} reading of preimmunization sera.

Because the design of the IC assay is based on a solid-phase capture format, immobilized anti-peptide antibody must be capable of binding native antigen in solution. For this purpose, IgG fractions of rabbit anti-HBs 125-137 and anti-HBe 73-87 were prepared and coated onto microtiter wells, and the ability to bind either HBsAg or HBeAg in solution was determined. The results are shown in Figure 1. Anti-HBs 125-137 quantitatively bound HBsAg but not HBeAg (Figure 1A), and reciprocally anti-HBe 73-87 bound HBeAg but not HBsAg (Figure 1B). The bound native antigens were detected with polyclonal human antibodies labelled with peroxidase. The fact that native antigen epitopes were still available for binding by the labelled antibodies suggested that the anti-peptide antibodies bound the native antigens at unique sites.

The absence of competitive binding between anti-native and anti-peptide antibodies is another characteristic that is required of an anti-peptide antibody that is to be used to capture immune-complexed antigen. As shown in Table II, rabbit

anti-HBs 125-137 binding to HBsAg was not significantly blocked by murine or human antibodies raised against native HBsAg. In contrast, the control rabbit anti-HBs antibody, raised against native HBsAg, was blocked by both murine and human polyclonal anti-HBs antisera. Similarly, anti-HBe 73-87 only minimally competed with polyclonal murine and human antibodies specific for the native HBeAg, whereas control rabbit anti-HBe was significantly blocked by the competitor antibodies (Table II). The minimal competition between anti-HBe 73-87 and antibodies raised against native HBeAg suggest a contribution of residues 73-87 in a native HBeAg epitope as previously suggested (Salfeld et al., J. Virol., 63:798 (1989). However, the minimal inhibition indicates that the linear peptide 73-87 does not fully represent an entire native epitope, which most likely requires greater conformational integrity. In support of the conformational dependence of the epitope(s) involving residues 73-87, none of the numerous HBcAg and HBeAg-specific monoclonal antibodies we have examined significantly bind to the 73-87 peptide.

Table II. Anti-peptide Antibodies Do Not Compete With Anti-HBs and Anti-HBe for Binding to Native Antigen

Rabbit Antibody	Competitor Antibody (% Inhibition)							
	Murine a-HBs		Murine a-HBe		Human a-HBs		Human a-HBe	
	1:25	1:100	1:25	1:100	1:25	1:100	1:25	1:100
a-HBs	40	34	0	0	72	42	0	0
a-HBs 125-1379	0	0	0	14	0	0	0	0
a-HBe	0	0	86	74	0	0	42	27
a-HBe 73-87	0	0	28	10	0	0	7	2

Solid-phase HBsAg or HBeAg (50 ng/ml) were incubated with dilutions of murine or human-derived competitor antibodies or normal serum followed by the addition of rabbit antibodies specific for the native antigens (a-HBs/a-HBe) or the peptide antigens. The data is expressed as percent inhibition by the competitor antibodies relative to the inhibition in the presence of normal murine or human serum.

Alternatively, the lack of competition between the antibodies raised against the peptide antigens and the antibodies raised against the native antigens (Table II) may be due to differences in antibody avidity. Because of the assumption that the anti-peptide antisera may be of lower avidity for the native antigens, the antibodies raised against the native antigens were used as the competitors. The failure of the higher avidity antibodies raised against the native antigens to prevent the binding of the anti-peptide antibodies to the native antigens indicate that differences in antibody avidity do not account for the lack of competition. Furthermore, the antibody competition experiments were performed in

both directions and yielded similar results. Therefore, the selected anti-peptide antibodies bind the respective native antigens at unique sites which do not significantly interfere with simultaneous binding by antibodies raised against the native proteins.

7. Use of Solid-Phase Anti-HBe 73-87 To Detect Preformed HBeAg/Anti-HBe Immune Complexes

The ability of solid-phase anti-HBe 73-87 to efficiently bind native HBeAg in solution and to bind at a unique site not competitive with antibodies raised to the native HBeAg, suggested that anti-HBe 73-87 may represent an ideal reagent to bind HBeAg complexed to human anti-HBe. To test this method, HBeAg/anti-HBe immune complexes were prepared using recombinant HBeAg and human polyclonal anti-HBe antibodies at varying antigen and antibody concentrations. Solid-phase anti-HBe 73-87 was used as the capture antibody and bound HBeAg/anti-HBe IC's were detected with a peroxidase-labelled monoclonal anti-human IgG reagent. Analysis of a wide range of HBeAg concentrations and at varying anti-HBe dilutions (i.e., checkerboard analysis) allowed the sensitivity of the IC assay to be determined in conditions of antigen or antibody excess. The HBeAg concentrations were chosen to approximate the range of serum concentrations of HBeAg during HBV infection. The results are shown in Table III.

Table III. Use of solid-phase anti-HBe 73-87 to detect preformed HBeAg/anti-HBe immune complexes.

	HBeAg (lg/ml)								
	0	.0006	.003	.01	.04	.16	.64	2.5	10	40
Human a-HBe (dilution)										
0	0	.01	.01	.01	0	0	.01	.01	.01	0
1:2560	0	0	0	.02	.07	.06	.09	0	0	0
1:512	0	.03	.05	.08	.16	.29	.28	.15	.13	.03
1:128	0	.04	.13	.15	.31	.62	.67	.78	.27	.20
1:32	0	.04	.18	.27	.39	.78	1.1	.95	.46	.16
1:8	0	.07	.16	.22	.20	.61	.91	.87	.50	.16
1:32	0	0	0	0	0	.01	0	0	0	0
1:8	0	0	1	0	0	.02	.03	.02	.02	.01

The indicated concentrations of soluble HBeAg were mixed with equal volumes of dilutions of a polyclonal human anti-HBe or normal human serum (NHS), and the preformed (ICs) were allowed to equilibrate overnight at 4°C. The HBeAg/anti-HBe ICs were then added to plastic wells coated with an IgG fraction of anti-HBe 73-87. The bound HBeAg/anti-HBe ICs were detected by a peroxidase-labelled Mab specific for human Ig. The data are expressed as absorbance (OD_{492}) corrected for the absorbance value in the absence of HBeAg for each antibody dilution. The boxed values are at least 3x the NHS background.

As shown in Table III, the solid-phase assay efficiently detected HBeAg/anti-HBe IC's over a wide range of antigen and antibody concentrations. As expected, the sensitivity of the solid-phase IC assay varied as a function of HBeAg concentration at a given

anti-HBe dilution. For example, at an anti-HBe dilution of 1:2560 only a concentration of 0.64 ug/ml of HBeAg yielded detectable IC's, at an anti-HBe dilution of 1:512 IC's were detected over a range of HBeAg concentrations (0.04-10.0 ug/ml), and at anti-HBe dilutions between 1:8 and 1:128 IC's were detected at HBeAg concentrations from 0.003 to 40 ug/ml. Also note that optimum IC detection occurred at intermediate HBeAg concentrations (.64-2.5 ug/ml) and IC detection declined at either lesser or greater HBeAg concentrations (Table III). Furthermore, maximum IC detection occurred at an anti-HBe dilution of 1:32 and not at the lower dilution of 1:8. These results are consistent with the dependence of HBeAg/anti-HBe IC formation on the antigen/antibody ratio and the optimum ratio is intermediate between antibody excess and antigen excess. The optimal HBeAg concentration was 0.64 ug/ml in the context of an anti-HBe dilution of 1:32.

It was also of interest to determine the ability of a direct solid-phase anti-HBe assay to detect "free" anti-HBe in the presence of soluble HBeAg. The direct assay utilized HBeAg (50 ng/well) as the solid-phase ligand and a peroxidase-labelled monoclonal anti-human IgG as the second antibody. The preformed HBeAg/anti-HBe IC's utilized in the solid-phase IC assay were assayed for anti-HBe activity in the direct EIA and by a commercial anti-HBe assay (Abbott) (Table IV).

Table IV. Sensitivity of Direct Solid-Phase Anti-HBe Assay in the Presence of Soluble HBeAg

	HBeAg (lg/ml)									
	0	.0006	.003	.01	.04	.16	.64	2.5	10	40
Human a-HBe (dilution)										
0	0	0	0	0	0	0	0	0	0	0
1:512	.15^{-a}	.07	.10	.08	.04	0	0	0	0	0
1:128	.77^{+}	.53^{-}	.59^{-}	.58^{-}	.46^{-}	.18^{-}	.03	.01	.01	0
1:32	1.2^{+}	1.1^{+}	1.1^{+}	1.0^{-}	.92^{-}	.65^{-}	.23^{-}	.04	.02	.02
1:8	1.7^{+}	1.6^{+}	1.4^{+}	1.4^{+}	1.3^{+}	1.1^{-}	.87^{-}	.28^{-}	.06	.05

Dilutions of a polyclonal human anti-HBe serum or normal human serum (NHS) were mixed with an equal volume of buffer or the indicated concentrations of soluble HBeAg and allowed to equilibrate overnight at 4°C. The ability to detect free anti-HBe in the mixtures was determined by ELISA. Solid-phase HBeAg (50 ng/well) served as the ligand and a peroxidase-labelled Mab anti-human Ig was used as the second antibody.

[a]The superscripts refer to the absence ($^{-}$) or presence ($^{+}$) of anti-HBe as determined by the commercial (Abbott) anti-HBe assay. The values to the left of the line are at least 3x the NHS background.

"Free" anti-HBe was detected by the direct EIA assay as a function of the initial anti-HBe dilution and the soluble HBeAg concentration, as expected. At an anti-HBe dilution of 1:32, an HBeAg concentration of 2.5 ug/ml (125 ng) was sufficient to neutralize the detection of anti-HBe by the direct EIA, whereas, an HBeAg concentration of 0.64 ug/ml (32 ng) was only partially inhibitory. This is consistent with approximately equivalent binding of anti-HBe to HBeAg

on the solid-phase and to HBeAg in solution inasmuch as ~50 ng of HBeAg was present on the solid-phase. Note that the solid-phase IC assay appears more sensitive in the context of high HBeAg concentrations as compared to the direct EIA, suggesting that anti-HBe is not able to dissociate from HBeAg complexes and bind solid-phase HBeAg, at least at higher antigen concentrations. Also note that the direct anti-HBe EIA as well as the solid-phase IC assay is significantly more sensitive than the commercial anti-HBe assay. Most likely this results from the fact that the commercial anti-HBe assay is a competitive EIA.

8. Use of Solid-Phase Anti-HBs 125-137 to Detect Preformed HBsAg/Anti-HBs Immune Complexes

Similar to the results obtained with anti-HBe 73-87, solid-phase anti-HBs 125-137 efficiently captured HBsAg complexed to human polyclonal anti-HBs over a range of antigen/antibody ratios (Table V). At an anti-HBs dilution of 1:8 HBsAg/anti-HBs IC's were detected at HBsAg concentrations from 0.01 ug/ml to 40 ug/ml. An HBsAg concentration of 100 ug/ml appeared to represent a level of antigen excess which precluded IC detection in this assay.

Table V. Use of Solid-Phase Anti-HBs 125-137 to Detect Preformed HBsAg/anti-HBs Immune Complexes

	HBsAg (µg/ml)								
	0	.01	.04	.16	.64	2.5	10	40	100
Human a-HBs (dilution)									
0	0	0	0	.01	0	0	0	0	0
1:512	0	.01	.01	.03	.04	.03	.01	0	0
1:128	0	.04	.04	.08	.19	.18	.16	.12	0

1:32	0	.11	.24	.34	.35	.37	.28	.14	0
1:8	0	.22	.24	.22	.21	.24	.83	.39	0
NHS									
1:32	0	0	0	0	0	0	0	0	0
1:8	0	0	.01	.02	0	.03	0	0	0

The indicated concentrations of soluble HBsAg were mixed with equal volumes of dilutions of a polyclonal human anti-HBs or normal human serum (NHS), and the preformed immune complexes (ICs) were allowed to equilibrate overnight at 4°C. The HBsAg/anti-HBs ICs were then added to plastic wells coated with an IgG fraction of anti-HBs 125-137. The bound HBsAg/anti-HBs ICs were detected by a peroxidase-labelled Mab specific for human Ig. The data are expressed as absorbance (OD_{492}) corrected for the absorbance value in the absence of HBsAg for each antibody dilution. The boxed values are at least 3x the NHS background.

However, the HBsAg/anti-HBs IC assay was clearly more sensitive than a direct binding anti-HBs assay, which was not able to detect "free" anti-HBs at HBsAg concentrations from 0.64 to 40 ug/ml depending on the initial anti-HBs dilution (Table VI).

Table VI. Sensitivity of Direct Solid-Phase Anti-HBs Assay in the Presence of Soluble HBsAg

	HBsAG (µg/ml)								
	0	.01	.04	.16	.64	2.5	10	40	100
0	0	0	0	0	0	0	0	0	0
1:512	.35[+a]	.34[+]	.27[+]	.15[-]	.02	01	01	0	0
1:128	1.1[+]	1.0[+]	.90[+]	.65[+]	.32[+]	.03	.02	0	0
1:32	1.7[+]	1.6[+]	1.5[+]	1.5[+]	1.0[+]	.52[+]	.06	.02	0
1:8	1.9[+]	1.8[+]	1.7[+]	1.8[+]	1.7[+]	1.0[+]	0.8[+]	.12	0

Dilutions of a polyclonal human anti-HBs serum or normal human serum (NHS) were mixed with an equal volume of buffer or the indicated concentrations of soluble HBsAg and allowed to equilibrate overnight at 4°C. The ability to detect free anti-HBs in the mixtures was determined by ELISA. Solid-phase HBsAg (50 ng/well) served as the ligand and a peroxidase-labelled Mab anti-human Ig was used as the second antibody.

[a]The superscripts refer to the absence ($^-$) or presence ($^+$) of anti-HBs as determined by the commercial (Abbott) anti-HBs assay. The values to the left of the line are at least 3x the NHS background.

9. Evaluation of Clinical Specimens Using the Solid-Phase IC Assays

Although the anti-peptide based, solid-phase IC assays performed well using premixed IC's, a useful IC assay must detect "natural" IC's as they exist in human sera. Therefore, the sera of 20 patients diagnosed with HBsAg-positive, HBeAg-positive, chronic active hepatitis (CAH) B were compared with the sera of 20 healthy control subjects for the presence of HBeAg-containing immune complexes (HBeAg/IC's) and HBsAg-containing immune complexes (HBsAg/IC's) (Table VII).

Table VII. Detection of Anti-HBs and Anti-HBe and Immune Complexes in Sera of Chronic Active Hepatitis B Patients Seronegative by Commercial Assay

Assay	Chronic Active Hepatitis (HBeAg+/HBsAg+)	Healthy Controls n = 20

n = 20

Anti-HBe (Direct EIA)	20/20[a] (0.45 % 0.2)	0/20 (.019 % .011)
Anti-HBe (Abbott)	0/20	0/20
HBeAg/ICs	8/20[a] (0.46 % 0.14)	0/20 (.17 % .07)
Anti-HBs (Direct EIA)	7/20[b] (0.41 % 0.34)	0/20 (.08 % .05)
Anti-HBs (Abbott)	0/20	0/20
HBsAg/ICs	9/20[a] (0.3 % 0.07)	0/20 (.11 % .05)

The sera of 20 patients with HBsAg +, HBeAg + chronic active hepatitis B were compared with the sera of 20 healthy control subjects in the indicated assays. The commercial (Abbott) anti-HBe and anti-HBs assays were performed according to the manufacturers instructions using undiluted sera. The direct EIA methods used to detect anti-HBe and anti-HBs, and the methods to detect antigen-specific immune complexes (ICs) are described herein. The direct EIA's and IC assays utilized a 1:50 dilution of sera. The number of positive sera/number tested and the mean absorbance (OD_{492}) plus s.d. are shown. An OD_{492} value greater than the mean plus 2 s.d. of the absorbance of 20 control sera was designated as positive.

[a]CAH vs. controls, $p < .01$.

[b]CAH vs. controls, $p < .05$

Although all CAH patients were seronegative for anti-HBe and anti-HBs by commercial assays (Abbott), the direct anti-HBe EIA revealed that 20 of 20 CAH sera actually possessed anti-HBe reactivity, and the direct anti-HBs EIA detected 7 of 20 anti-HBs positive

samples. This finding is consistent with another recent report that anti-HBe can co-exist with HBeAg in a proportion of CAH patients and can be detected with a sensitive solid-phase anti-HBe assay (Sallberg et al., 1991). Eight of 20 CAH patient sera also exhibited HBeAg-containing IC's as measured by the solid-phase IC method utilizing anti-HBe 73-87 as the capture reagent. The failure to detect HBeAg/IC's in a number of specimens containing both HBeAg and anti-HBe may have been due to low HBeAg concentrations (≤ 10 ug/ml) since sera were diluted 1:50 for the IC assay due to occasional high backgrounds at lower dilutions. We anticipate that the sensitivity of the HBeAg/IC assay will be optimized by the use of a monoclonal anti-peptide antibody instead of a rabbit IgG fraction on the solid-phase. The HBsAg-specific IC assay detected HBsAg/IC's in 9 of the 20 CAH patient specimens, whereas, anti-HBs was measured in 7 of 20 by the direct EIA method. The greater sensitivity of the HBsAg/IC assay as opposed to the direct anti-HBs EIA may reflect the relatively high serum concentrations of HBsAg (≤ 100 ug/ml) in these CAH patients, which adversely affects the direct EIA, and may actually enhance the sensitivity of the IC assay (Tables V and VI). Cumulatively, the combined use of the direct EIA's and the antigen-specific, solid-phase IC assays revealed the presence of active humoral immune responses occurring in these CAH patients, which are not apparent from analysis with the conventional serological assays available commercially.

10. <u>Evaluation of the Kinetics of Seroconversion and Immune Complex Formation</u>

Chronic HBV infection is not a static process,

and a major advantage to the development of antigen-specific IC assays is the ability to monitor the specificity and progression of the patients underlying immune response. This information may aid in the prognosis and/or recommendation for treatment of CAH patients. The course of a CAH patient (N.Y.) monitored for a 3 year period is shown in Figure 2. The conventional serological analysis of patient N.Y. is illustrated in the upper panel (Figure 2A). The patient was HBV DNA-positive and HBeAg-positive through 4/82 and HBsAg-positive throughout the observation period; and seronegative for anti-HBe until 3/83 and seronegative for anti-HBs throughout as measured by commercial antibody assays. In contrast, analysis for circulating IC's and direct antibody analysis revealed significant HBsAg/IC's as early as 6/81 co-existent with low level anti-HBs, which increased until HBV DNA clearance and then declined rapidly (Figure 2B, middle panel). Similarly, this patient evidenced significant anti-HBe antibody production as early as 6/81, which persisted throughout the observation period, and low level HBeAg/IC's (Figure 2C, bottom panel).

A second CAH patient (E.I.) who possessed similar conventional serology as patient N.Y. (Figure 3A, upper panel), demonstrated HBeAg/IC's and anti-HBe antibodies 5 years prior to seroconverting to the HBeAg-negative, anti-HBe-positive phase as determined by conventional assays (Figure 3B, bottom panel). Note the decline of HBeAg/IC's at the time of HBV DNA clearance and the reciprocal rise in free anti-HBe. Resolution of HBV infection always appears to be preceded by the appearance of antibodies to HBeAg measured directly as free anti-HBe or by the presence of HBeAg/IC's (manuscript in preparation).

In summary, the antigen-specific IC assays of this invention represent valuable additions to the serological analysis of chronic HBV infection. This method of IC detection is clearly not limited to the HBV system. It is anticipated that the solid-phase IC method may be applied to the detection of IC's containing other pathogen-derived or self antigens provided that suitable anti-peptide antibodies with the requisite characteristics can be developed. The necessary characteristics simply are that the anti-peptide antibody must bind soluble native antigen efficiently, and must not compete for binding with antibody raised against the native protein. Antibodies with these characteristics can be produced by methods other than immunization with synthetic peptides. For example, immunizations with denatured antigen may also result in the production of non-competing, native-reactive antibodies. However in this circumstance the production of monoclonal antibodies would be required because of the polyclonal nature of the response. The preselected specificity of an anti-peptide response may preclude the necessity for monoclonal antibody production.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Scripps Research Institute
(B) STREET: 10666 North Torrey Pines Road
(C) CITY: La Jolla
(D) STATE: CA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 92037
(G) TELEPHONE: 619-554-2937
(H) TELEFAX: 619-554-6312

(ii) TITLE OF INVENTION: METHODS FOR THE DETECTION OF ANTIGEN-SPECIFIC IMMUNE COMPLEXES

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
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(A) APPLICATION NUMBER: US 07/961,119
(B) FILING DATE: 14-OCT-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys

1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Val Asn Leu Glu Asp Pro Ala Ser Arg Asp Leu Val Val Ser Cys
1 5 10 15

What is Claimed is:

1. A method for detecting the presence of an immunocomplex having antibody molecules complexed with a preselected antigen in a sample comprising the steps of:

a) admixing the sample with a detecting antibody molecule to form an immunoreaction admixture, said detecting antibody molecule capable of immunoreaction with said immunocomplex at a preselected site of said immunocomplex;

b) maintaining said immunoreaction admixture under immunoreaction conditions sufficient for said detecting antibody molecule to immunoreact with said immunocomplex and form a detecting antibody-immunocomplex reaction product;

c) detecting the presence of said detecting antibody-immunocomplex reaction product, and thereby said immunocomplex.

2. The method of claim 1 wherein said detecting antibody molecule is in the solid phase.

3. The method of claim 1 wherein said sample is a body fluid sample selected from the group consisting of blood and serum.

4. The method of claim 1 wherein said preselected antigen is selected from the group consisting of bacterial, viral, parasitic, fungal, tumor and self antigen.

5. The method of claim 4 wherein said viral antigen is selected from the group of viruses consisting of hepatitis B virus (HBV), human immunodeficiency virus, influenza A virus, Epstein-Barr virus, togavirus and rubella virus.

6. The method of claim 5 wherein said HBV antigen is HBsAg or HBeAg.

7. The method of claim 1 wherein said detecting

antibody immunoreacts with the hepatitis B virus (HBV) antigen HBsAg and immunoreacts with a polypeptide having an amino acid residue sequence shown in SEQ ID NO 1.

8. The method of claim 1 wherein said detecting antibody immunoreacts with the hepatitis B virus (HBV) antigen HBeAg and immunoreacts with a polypeptide having an amino acid residue sequence shown in SEQ ID NO 2.

FIG. 1A

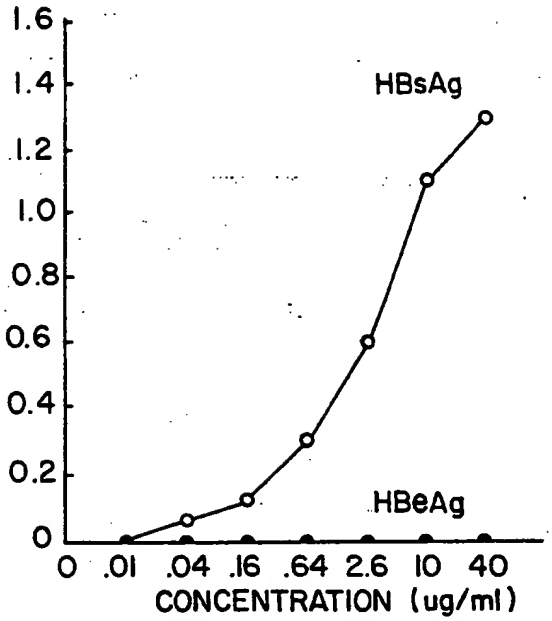

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
HBsAg
HBeAg
0 .01 .04 .16 .64 2.6 10 40
CONCENTRATION (ug/ml)

FIG. 1B

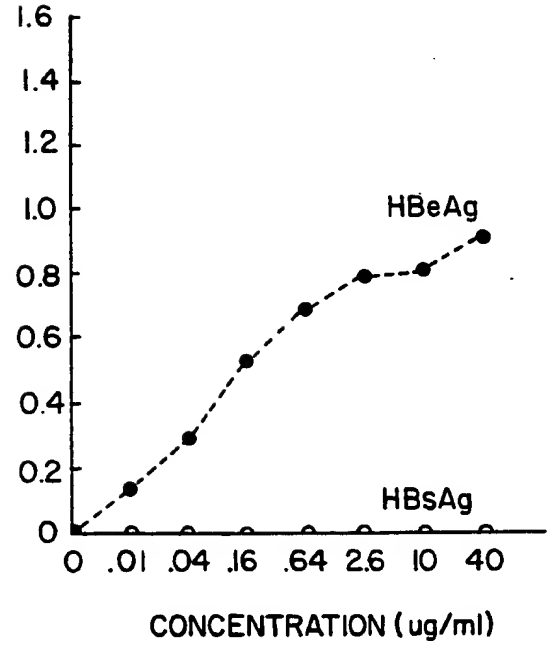

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
HBeAg
HBsAg
0 .01 .04 .16 .64 2.6 10 40
CONCENTRATION (ug/ml)

FIG. 2A

HBV DNA	+	+	+	+	+		±	-	-	-
HBeAg	+	+	+	+	+		-	-	-	-
a-HBe (Abbott)	-	-	-	-	-		-	+	±	+
HBsAg	+	+	+	+	+		+	+	+	+
a-HBs (Abbott)	-	-	-	-	-		-	-	-	-
ALT	19	22	3	54	2		65	3	8	4

FIG. 2B

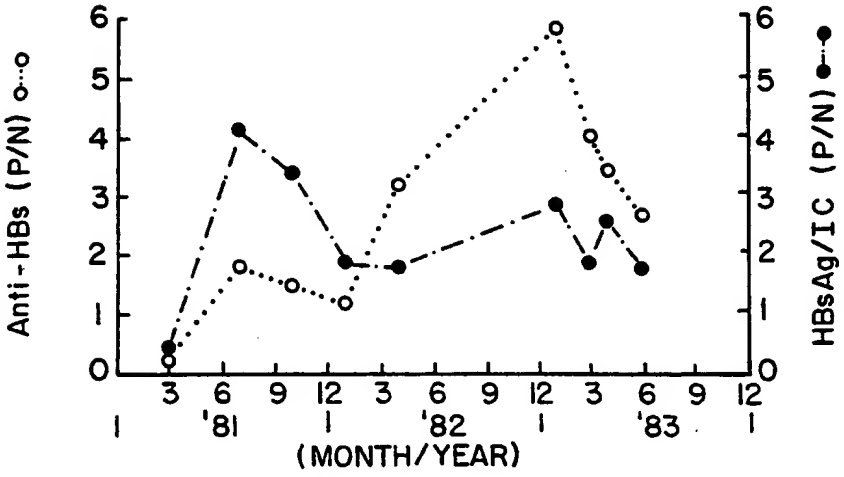


FIG. 2C

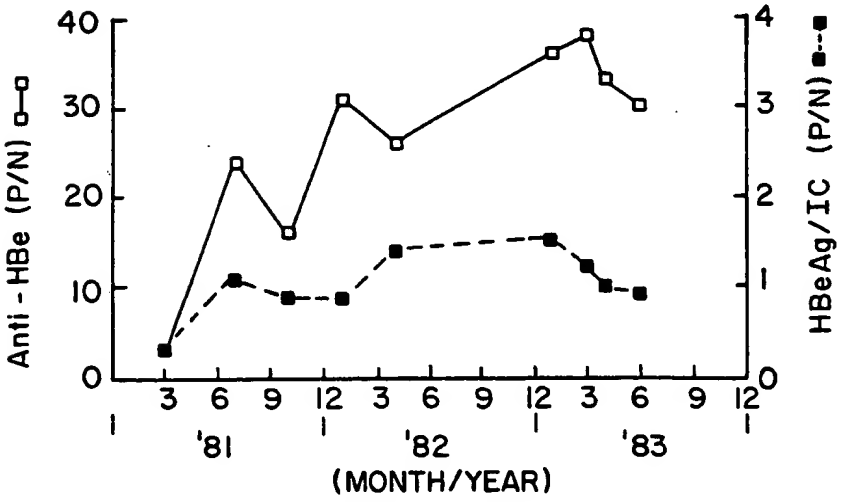
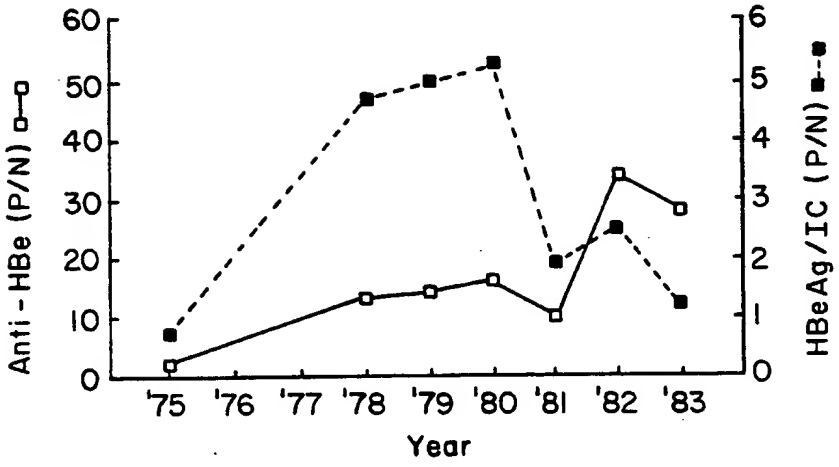


FIG. 3A

HBV DNA	++	+++	++	++	+	−	−
HBeAg	+	+	+	+	±	−	−
a-HBe (Abbott)	-	-	-	-	-	-	+
HBsAg	+	+	+	+	+	+	+
a-HBs (Abbott)	-	-	-	-	-	-	-
ALT	14	4	9	8	42	27	11

FIG. 3B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09903

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.1, 7.22, 7.23, 7.31, 7.92; 436/506, 507, 518, 820, 528; 530/387.9, 388.3, 389.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, CA; A-GeneSeq 12, PIR-38, Swiss-Prot 26
search terms: immune complex?, hepatitis, hbv, ebv, hiv, rubella, parasit?, influenza, epitope#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	CLINICAL AND EXPERIMENTAL IMMUNOLOGY, Volume 72, issued 1988, S. Rath et al, "IgG Subclass Composition of Antibodies to HBsAg in Circulating Immune Complexes From Patients with Hepatitis B Virus Infections", pages 164-167, see entire document, especially page 165, Col 1.	1-6 7,8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 January 1994	Date of mailing of the international search report 26 JAN 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JAMES L. GRUN, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09903

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 79, issued September 1982, D.A. Shafritz et al, "Monoclonal Radioimmunoassays for Hepatitis B Surface Antigen: Demonstration of Hepatitis B Virus DNA or Related Sequences in Serum and Viral Epitopes in Immune Complexes", pages 5675-5679, see entire document, especially page 5677, Col 1.	7
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 78, Number 2, issued February 1981, J.R. Wands et al, "Immunodiagnosis of Hepatitis B with High-Affinity IgM Monoclonal Antibodies", pages 1214-1218, see pages 1214-1215, especially Table 1.	7
Y	JOURNAL OF VIROLOGY, Volume 63, Number 2, issued February 1989, J. Salfeld et al, "Antigenic Determinants and Functional Domains in Core Antigen and e Antigen from Hepatitis B Virus", pages 798-808, see entire document, especially page 804, Col 1.	8
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Volume 79, issued July 1982, P.K. Bhatnagar et al, "Immune Response to Synthetic Peptide Analogues of Hepatitis B Surface Antigen Specific for the a Determinant", pages 4400-4404, see entire document, especially page 4402, Table 2.	7
Y	NATURE, Volume 281, issued 25 October 1979, F. Galibert et al, "Nucleotide Sequence of the Hepatitis B Virus Genome (Subtype ayw) Cloned in E. coli", pages 646-650, see especially Figure 3.	8
Y	EP, A, 0,044,710 (LERNER ET AL) 27 January 1982, see entire document.	7,8
X	US, A, 4,544,640 (SOMA ET AL) 01 October 1985, see entire document, especially: Col 1-4; Col 8, lines 38-68; Claims 2, 4, 22.	1-4